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Ubiquitous Transcriptional Pausing Is Independent of RNA Polymerase Backtracking

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Summary

RNA polymerase (RNAP) transcribes DNA discontinuously, with periods of rapid nucleotide addition punctuated by frequent pauses. We investigated the mechanism of transcription by measuring the effect of both hindering and assisting forces on the translocation of single *Escherichia coli* transcription elongation complexes, using an optical trapping apparatus that allows for the detection of pauses as short as one second. We found that the vast majority of pauses are brief (1–6 s at 21°C, 1 mM NTPs), and that the probability of pausing at any particular position on a DNA template is low and fairly constant. Neither the probability nor the duration of these ubiquitous pauses was affected by hindering or assisting loads, establishing that they do not result from the backtracking of RNAP along the DNA template. We propose instead that they are caused by a structural rearrangement within the enzyme.

Introduction

The rate at which RNAP adds nucleotides to the 3′ end of a growing RNA transcript is highly nonuniform. The time required for nucleotide addition increases by an order of magnitude or more over average during events that have been termed "transcriptional pauses" (Kassavetis and Chamberlin, 1981; Reisbig and Hearst, 1981; Kadesch and Chamberlin, 1982; Winkler and Yanofsky, 1981; Levin and Chamberlin, 1987; Matsuzaki et al., 1994; Lyakhov et al., 1998).

Elucidating the mechanisms of pausing is vital for several reasons. First, pausing plays important roles in transcriptional regulation, for example, in synchronizing interactions of ribosomes or transcription factors with RNAP movements (reviewed in Chan and Landick, 1994; Landick and Yanofsky, 1987; Richardson and Greenblatt, 1996; Uptain et al., 1997). Second, pausing represents the first event in transcriptional termination (Kassavetis and Chamberlin, 1981; McDowell et al., 1994; Yin et al., 1999; Gusarov and Nudler, 1999). Both pausing and termination are suppressed by elongation factors that interact with the transcription elongation complex (TEC) (e.g., λN and λQ; Richardson and Greenblatt, 1996; Roberts et al., 1998; Weisberg and Gottesman, 1999; and references therein). Finally, frequent pausing is observed during the transcription of genomic DNA (Kassavetis and Chamberlin, 1981; Matsuzaki et al., 1994; Adelman et al., 2002). For bacterial RNAP, such pauses are thought to limit the overall rate of transcription, thereby synchronizing transcription with translation (translating ribosomes release paused RNAPs), and allowing Rho-catalyzed termination of transcription should translation fail (Landick et al., 1985; Richardson and Greenblatt, 1996).

Both bulk (Kassavetis and Chamberlin, 1981; Theissen et al., 1990; Matsuzaki et al., 1994; Wang et al., 1995) and single-molecule (Davenport et al., 2000; Adelman et al., 2002; Forde et al., 2002) transcription experiments demonstrate that, in most cases, only a fraction of RNAP molecules pause at any given DNA sequence. Thus, a pause is characterized by two empirical parameters: the efficiency (equivalently, the pause probability or frequency) and the lifetime (equivalently, the pause duration or half-life). The observation that pause efficiency is generally less than 100% is consistent with a branched reaction pathway where pauses occur by reversible isomerization of the TEC into a catalytically inactive state that does not occur during the cycle of ordinary elongation.

To date, only high-efficiency pauses with demonstrated roles in transcription regulation have been characterized in mechanistic detail (Roberts et al., 1998; Artsimovitch and Landick, 2000; Palangat and Landick, 2001, and references therein). These pauses fall into at least two classes, both of which arise from interactions of RNAP with specific nucleic acid sequences, including those found in duplex DNA downstream of the RNAP active site, in the active site itself, or in the RNA:DNA heteroduplex upstream of the active site (Korzheva et al., 2000; Gnatt et al., 2001; Landick, 2001).

In one class of pauses, a nascent RNA hairpin structure is associated with the paused conformation of the enzyme. Here, pausing is thought to be generated by a rearrangement in the active site that is stabilized by an allosteric interaction of the hairpin with the RNAP (Toulokhonov et al., 2001). A second class of pauses has been termed "backtrack" pausing, because it appears to be associated with rearward motion of RNAP along the DNA and RNA chains (Komissarova and Kashlev, 1997a; Nudler et al., 1997; Landick, 1997; Nudler, 1999; Artsimovitch and Landick, 2000). Backtracking has been demonstrated at sites of transcriptional arrest and when RNAPs are artificially halted by the removal of nucleoside triphosphates (NTPs) (Komissarova and Kashlev, 1997b; Palangat and Landick, 2001; Reeder and Hawley, 1996; Toulme et al., 1999).

During backtracking, the 3′ end of the nascent RNA protrudes from a pore in the enzyme thought to be the

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NTP-entry channel (Fu et al., 1999; Zhang et al., 1999). Backtracked TECs maintain the register between the DNA and RNA strands, but are catalytically incompetent because the 3′ end of the RNA is displaced from the active site. Backtracking may also be responsible for the comparatively large-scale motion (~8–9 bp) of RNAP relative to DNA inferred from the stalling behavior of single TECs under applied loads (Wang et al., 1998). In such stalling experiments, increasing tension is applied between the RNAP and the downstream DNA end, which destabilizes the catalytically active form of the TEC relative to the backtracked form.

One proposal is that a short (e.g., single nucleotide), reversible backtracking event would induce a brief pause, whereas a larger backtracking motion would lead to lengthier pausing, or possibly to irreversible transcriptional arrest (Komissarova and Kashlev, 1997a; Nudler et al., 1997; Landick, 1997). However, a causal connection between backtracking and most instances of pausing remains to be established, because RNAP backtracking has only been shown in special circumstances, e.g., under conditions of transcriptional arrest (Nudler et al., 1997), a complete absence of NTPs (Komissarova and Kashlev, 1997a, 1997b), or during GreA-stimulated cleavage of the nascent RNA (Artsimovitch and Landick, 2000; Marr and Roberts, 2000).

We performed single molecule, feedback-enhanced optical trapping experiments to characterize the mechanism of pausing during transcription of the *rpoB* gene of *E. coli*. These experiments allowed us to detect and measure the duration of thousands of individual pause events at high spatiotemporal resolution. Our experiments were conducted over a wide range of external forces applied to the TEC, in orientations that either assisted or hindered the downstream motion of RNAP along DNA. The goal of applying force is to affect specifically the rate and equilibrium constants of conformational transitions—such as backtracking—responsible for RNAP movement relative to the template. For simple models of motion, the expected changes arising from applied force are straightforward to calculate from theory (Wang et al., 1998; Tinoco and Bustamante, 2002). Enzyme behavior under the application of load therefore constitutes a highly sensitive determination as to whether pauses may be generated by backtracking motions.

Results

RNAP Molecules Alternate between Constant-Velocity Transcription and Pausing

The single-molecule optical trapping experiment is illustrated in Figure 1A. Stalled TECs were specifically attached to avidin-coated polystyrene beads. The upstream end (for assisting force) or downstream end (for hindering force) of the template DNA was attached to the surface of a flow cell by means of an antibody linkage. Transcription was then restarted by perfusing the flow cell with 1 mM NTPs. The tethered bead was then trapped, and the attachment point of the tether to the surface was determined (Wang et al., 1997). Transcriptional elongation was measured in one of three different instrument modes. The majority of the records was recorded in a force-clamp mode, in which a constant force was maintained on RNAP by moving the stage to compensate for motion of the enzyme along the DNA. Using this technique, transcription can be measured with high resolution over long distances at a single, well-defined force. In other records, the force was varied dynamically on individual molecules, either to prevent further motion of the enzyme (position-clamp mode) or to subject the enzyme to a linearly increasing force (force-ramp mode).

The movement of RNAP was frequently punctuated by pauses of variable duration (Figure 1B). To measure the pausing kinetics of single molecules, individual traces were smoothed to reduce noise from Brownian motion and then differentiated to obtain the instantaneous velocity (Figure 1B, lower image; Experimental Procedures). The velocities of individual RNAP molecules typically displayed a bimodal distribution (e.g., Figure 1B, left inset and Figure 2A), with one peak corresponding to the rate of transcription between pauses and a second peak, near 0 bp/s, due to the pauses themselves. Histograms of velocity for each RNAP molecule were well fit by the sum of two Gaussians. We found no evidence for any additional velocity states in any of the individual records ($N = 143$). The transcription run velocity was calculated as the difference between the centers of the two Gaussians; this measure corrects for any slow drift in the apparatus and supplies the average speed of RNAP between detected pauses. Operationally, a pause was scored whenever the instantaneous velocity (Figure 1B) dropped below half the transcription run velocity.

Velocity between Pauses Differs from Molecule to Molecule

Whereas each individual RNAP molecule appeared to move at a single fixed speed, their individual speeds varied across the population of molecules (Figure 2A). The average run velocity was independent of template position (correlation; $p < 0.01$; data not shown), in agreement with previous observations (Davenport et al., 2000). Run velocities measured under constant-force conditions showed no detectable correlation with force, provided that the force was lower than that required to stall the complex (data not shown; Davenport et al., 2000; Forde et al., 2002). We therefore pooled transcription run velocities from individual records to generate a global velocity distribution (Figure 2B). The global average run velocity was 9.7 ± 4.8 bp/s (mean ± SD) at 1 mM NTPs (21 ± 0.5°C). The distribution is broad, confirming the heterogeneity in transcription rates among molecules.

Translocation Step Is Not Rate Limiting at Low Load

The relationship between force and velocity was determined by dynamically varying the load applied to single molecules. The remarkable flatness of the force-velocity (*F-v*) curve implies that reaction steps involving translocation are not rate-determining below ~24 pN (Figure 3). The steep decrease in velocity near stall is consistent with the entry into stall being accompanied by a comparatively large displacement of the bead attachment point on the enzyme relative to the DNA. The data can be fit by a simple Boltzmann-type model involving a transition

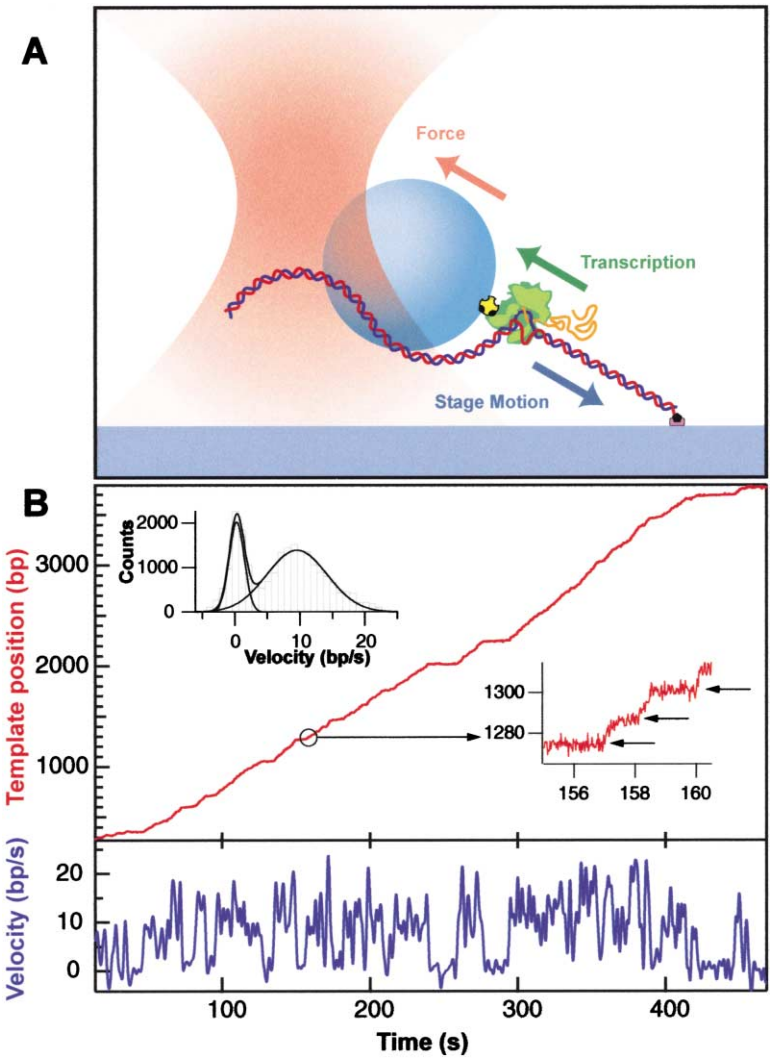

Figure 1. Single-Molecule Transcription Elongation

(A) Cartoon of the experimental geometry (not to scale). Transcribing RNA polymerase (green) with nascent RNA (gold) is attached to a polystyrene bead (blue) via a biotin-avidin (yellow) linkage. The upstream end of the duplex DNA (red/blue) is attached through a digoxigenin-antidigoxigenin linkage (violet) to the surface of a flowcell (blue) mounted on a piezoelectric stage. The bead is held in the optical trap (pink) at a predetermined position from the trap center, which results in a restoring force exerted on the bead (pink arrow). During transcription (green arrow), the position of the bead in the optical trap and hence the applied force is maintained by moving the stage both horizontally and vertically (blue arrow) to compensate for motion of the polymerase molecule along the template. For the geometry depicted, the applied force is in the direction of transcription (assisting load). When the digoxigenin label is placed instead on the downstream end of the DNA, the direction of transcription is reversed with respect to the trap, and the force opposes transcription (hindering load).

(B) Representative record of position (red) and velocity (blue) for a single polymerase molecule transcribing a 3.5 kbp template with 1 mM NTPs under 18 pN of hindering load. Pausing occurs on multiple timescales; distinct pauses of seconds-long duration can be seen in the traces, while shorter pauses ($\sim$1 s) can be discerned in the expanded portion of the trace (right inset; arrows). Negative values of instantaneous velocity are due to Brownian motion of the bead in the optical trap. The velocity distribution (left inset) is well fit by the sum of two Gaussians (pause = 0.28 $\pm$ 1.16 [s.d.] bp/s; run = 9.55 $\pm$ 4.64 bp/s). The transcription run velocity (see text) was 9.27 bp/s.

over an energy barrier, governed by a single distance parameter (Supplemental Data available at http://www.cell.com/cgi/content/full/115/4/437/DC1). This analysis suggests that RNAP could move by 9 $\pm$ 1 nt relative to the DNA upon entry into the stalled state, consistent with the 7.0–8.7 nt displacement estimated previously using the same model (Wang et al., 1998). However, the current measurements provide a much more stringent test of the model, which had previously been fit only to data on hindering forces applied to nonspecifically adsorbed RNAP molecules. It is important to note that the single-barrier model does not explain several features of load-induced stall. Many stalled complexes do not immediately recommence transcription following a rapid reduction in force, and some become irreversibly stalled (data not shown; Wang et al., 1998). These observations favor a scenario where some additional, slow reorganization must occur before the stall can be relieved. Additionally, the large heterogeneity in stall forces among complexes, and among multiple stalls for a single complex (data not shown; Wang et al., 1998), raises the possibility that individual stall events may be associated with rearward displacements of variable extent. Moreover, some complexes enter stall quite abruptly (data not shown), which is consistent with a discontinuous, sequence-dependent change in the energy barrier separating active and backtracked states. Despite the improved resolution and stability of the apparatus used for the present measurements, the noise in the position signal in position-clamp records remains somewhat larger than the $\sim$9 nt motion inferred for the stall event, precluding a direct observation of stall-induced backtracking.

Short, Ubiquitous Pauses in a Bacterial Gene

The distribution of pause lifetimes (Figure 4A) indicates that the vast majority of pauses were brief ($<$10 s). The average pause lifetime ($\sim$3 s) was similar to that measured in a recent single-molecule study (Adelman et al., 2002). However, the distribution of pause lifetimes out to 25 s was better fit by a sum of two exponentials than by a single exponential (*F*-test; $p < 10^{-5}$), implying that there are $\geq$2 species of pause complex. A small fraction of pauses ($<$5%) had durations in excess of 25 s, and these were not well fit by the double exponential (Figure

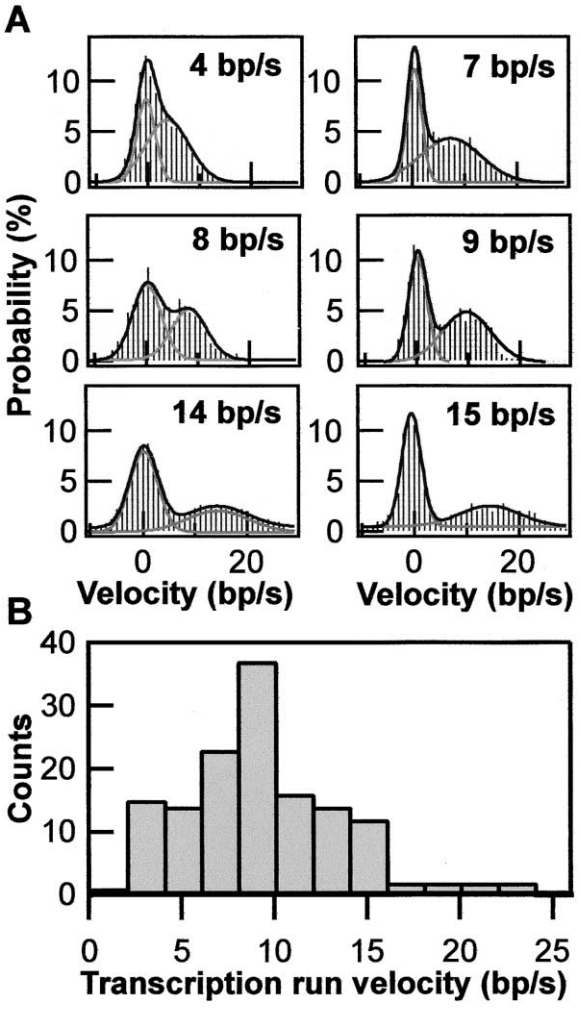

Figure 2. Transcription Rate Is Heterogeneous

(A) Normalized velocity distributions for individual single-molecule records. Histograms of velocity (see Figure 1B) are shown for 6 molecules at 1 mM NTPs under 14 pN hindering load, along with curve fits to the sum two Gaussians (solid lines). The transcription run velocity for each molecule is indicated.

(B) The distribution of transcription run velocities for the entire population of single molecule force-clamp records, spanning a range of force from −37 pN to 27 pN. The run velocity is 9.7 ± 0.4 bp/s (mean ± SE; $N = 143$).

4A, inset): such lengthy pauses were scored, but due to limited statistics were excluded from further analysis.

We next asked how pauses were distributed within the sequence of the template. Pauses occurred with comparable probability in every 100 bp segment, with a frequency varying between 0.5 ± 0.1 and 1.1 ± 0.1 per 100 bp (Figure 4B). The small but statistically significant variation in pausing as a function of template position suggests that pausing may be at least partly sequence-dependent. Thus, RNAP appears to have a low efficiency of pausing at multiple sequences distributed nearly uniformly over the template.

Pausing Is Not Affected by Force

It has been conjectured that most pauses are caused by backtracking, i.e., by the reverse translocation of

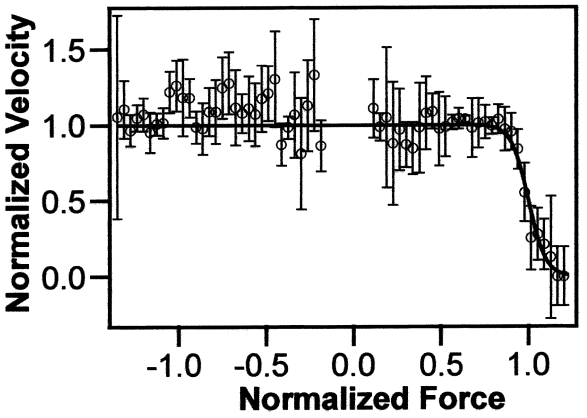

Figure 3. Single-Molecule Force-Velocity Relationship for RNA Polymerase

Normalized velocity (mean ± SD) versus normalized force (see Supplemental Data available on *Cell* website) at 1 mM NTP, fit to a single barrier model (line). Positive (negative) forces correspond to hindering (assisting) loads. Fit parameters: $a = 7.4 \pm 4.1 \times 10^8$; $\langle F_{1/2} \rangle = 27 \pm 2$ pN, corresponding to a displacement, δ, of 9 ± 1 bp.

RNAP over one or more nucleotides (Komissarova and Kashlev, 1997a; Nudler et al., 1997; Landick, 1997; Nudler, 1999). The backtracking hypothesis predicts that the equilibrium between the active and paused (backtracked) states should be shifted by forces that either assist or oppose translocation. In the most straightforward model, a single energy barrier separates the paused and elongation-competent states, and these states interconvert through a backtracking motion of RNAP relative to the DNA through some distance, *D* (Figure 5). The effective equilibrium constant for interconversion between the two states supplies the pause strength, *P*, which is the product of the pause probability and pause duration. The application of an external force, *F*, changes the free energy difference between paused and elongating states, and thus *P*, according to a Boltzmann factor (Wang et al., 1998; Tinoco and Bustamante, 2002):

$$P = P_0 \exp(FD/k_BT) \quad (1)$$

where P_0 is pause strength in the absence of force and k_BT is the thermal energy. The steep, exponential dependence of pause strength on load represents a testable prediction of the backtracking hypothesis. Measured pause strengths were essentially unaffected by either hindering or assisting loads from −37 to +27 pN (Figure 6). These data are inconsistent with the backtracking model, even for a backtracking distance as short as 1 bp. Backtracking distances >1 bp would only steepen the exponential dependence, resulting in curves that deviate still further from the experimental observations. Symmetric sliding models, in which the polymerase can enter a pause by sliding either backward or forward along the template, are equally inconsistent with our data; these produce a symmetric load dependence resembling the positive force portion of the Boltzmann exponential curve, but mirrored about zero load (Figure 6). Because the application of force alters the equilibrium between the paused and elongating positions of

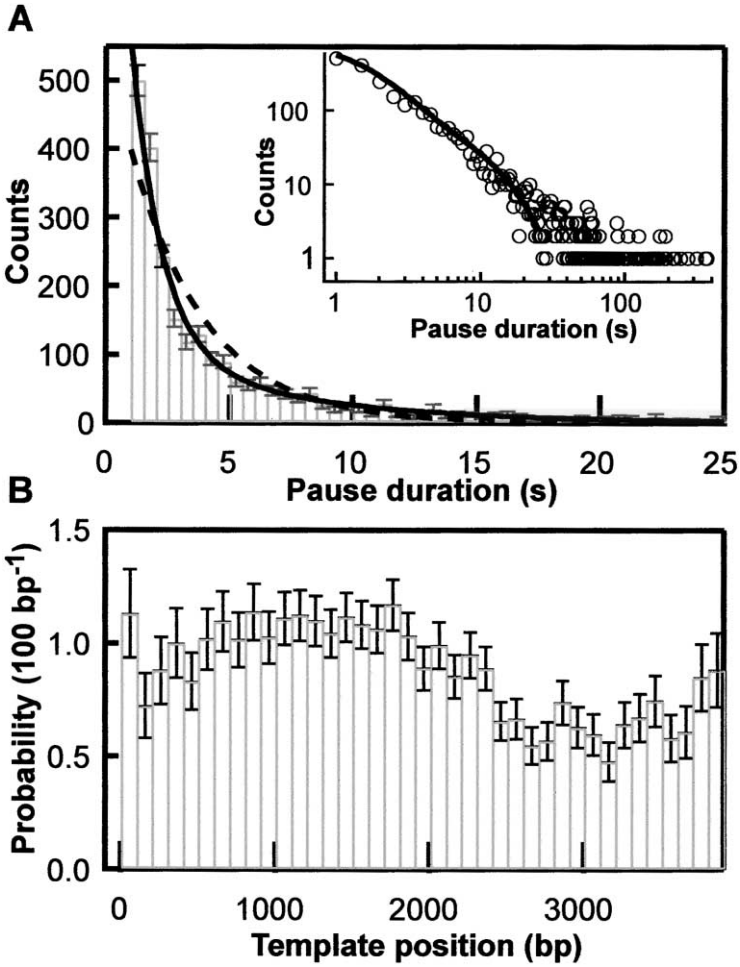

Figure 4. Transcriptional Pausing Is Ubiquitous

(A) Distribution of pause durations obtained in force-clamp mode for F over −37 to 27 pN at 1 mM NTPs, 21°C. The histogram of N = 2,432 pauses shorter than 25 s is better fit (F-test; $p < 10^{-5}$) by a sum of two exponentials (solid curve) than a single exponential (dashed curve), with lifetimes of 1.2 ± 0.1 s (amplitude 60 ± 3%) and 6.0 ± 0.4 s (amplitude 40 ± 3 %). Inset: double logarithmic plot of the complete pause distribution (N = 2,663) to 400 s and the same double exponential out to 25 s (solid curve).

(B) Pause probability density in force-clamp mode for F from −37 to 27 pN. The template was divided into 100 bp bins, and the pause probability was calculated for every bin (mean ± SD). The average pause probability per 100 bp bin was 0.87 ± 0.03 (SE).

the RNAP on the DNA, the expected dependence of pause strength on force is independent of the details of the transition between the two states. A simple, single energy barrier model is considered here, but the finding that P is independent of load also rules out backtracking for proposed models with more complex energy landscapes.

A recent single-molecule investigation of RNA polymerase (Forde et al., 2002) concluded that pausing was weakly force-dependent, in contrast to our findings. However, Forde and coworkers were restricted by experimental conditions to the analysis of a relatively rare class of extremely lengthy pauses (~100 s at 1 mM NTPs). Pauses of such duration represent <0.2% of the events reported here and are not described by the double exponential fit that accounts for the vast majority of the observed pauses (Figure 4A). These rare, very lengthy pauses likely occur through an entirely different mechanism.

Discussion

By examining the movement of single molecules of RNAP at high resolution under both assisting and opposing loads, we characterized the pausing behavior of RNAP during transcription of a bacterial gene in vitro. We find that at physiological NTP concentrations, RNAP transcriptional movement is dominated by a series of frequent, low-efficiency, short-duration pauses (Figure 4). These ubiquitous pauses are unaffected by load (Figure 6), hence cannot be explained by a mechanism involving the backtracking of RNAP, which has previously been proposed to be the dominant source of transcriptional pausing (Nudler et al., 1997; Komissarova and Kashlev, 1997a; Landick, 1997; Nudler, 1999; Epshtein and Nudler, 2003). We propose instead that these pauses correspond to a conformational change within RNAP, which may represent a so-called "unactivated intermediate," first described by Erie et al. (1993) in studies of nucleotide misincorporation.

Mechanisms of Ubiquitous, Backtracking-Independent Pausing

Several candidate mechanisms might explain low efficiency, backtrack-independent pausing, and we considered additional evidence in evaluating these possibilities. One potential source of pausing in single-molecule assays is competitive inhibition caused by the reversible binding of one of the three incorrect NTPs to the active site. We estimated the duration of such events using previously measured K_M and K_I values for RNAP (Supplemental Data available on *Cell* website). However, the

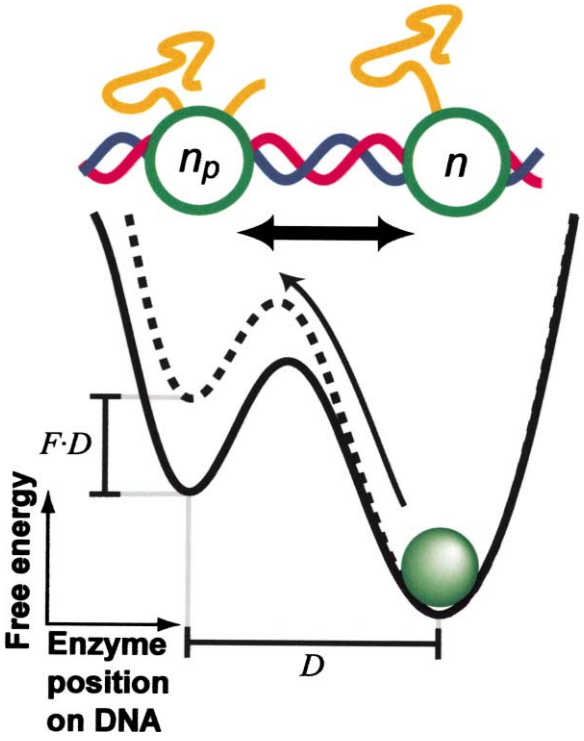

Figure 5. Free Energy Profile for Backtracking-Induced Pausing

The backtracking hypothesis proposes that a transient pause (state n_p) occurs when RNAP (green circle) slides backward by one or more bases (over distance D) along the DNA (red and blue). The resulting movement of the 3′-end of the nascent RNA (gold) relative to the polymerase active site prevents further transcription until the motion is reversed (returning to state n). The corresponding free energy diagram is a single barrier between two potential wells separated by a distance D. The application of external force alters the relative free energy of the states by the amount indicated (dashed line, no force; solid line, hindering force).

lifetimes of binding events predicted by our analysis are approximately an order of magnitude shorter than the durations of the ubiquitous pauses we measured. A second potential mechanism is NTP misincorporation, i.e., the covalent incorporation of noncomplementary nucleotides at the 3′ end of the nascent RNA. Although misincorporation could lead to pausing, it occurs at a frequency of 10^{-3} to 10^{-5} bp^{-1} in vitro (Erie et al., 1992), which is substantially lower than the pause density ($\sim 1.2 \times 10^{-2}$ bp^{-1}). Finally, it is formally possible that transcription continues uninterrupted during pauses via an "inchworming" mechanism, whereby some portion of RNAP advances while the remainder (to which the bead is attached) remains stationary (Chamberlin, 1994). However, given the observed pause times and translocation speeds measured, this mechanism would require the active site to move a sizable distance (many nm) relative to the C terminus of the β′ subunit, i.e., the point of attachment to the bead. Given the proximity of the C terminus to the active site and the large number of protein contacts between these regions (Zhang et al., 1999), any large-scale flexure seems difficult to reconcile with the known structure, and therefore unlikely. Furthermore, if pauses reflected such a nonmonotonic motion of the polymerase, then the translocation speeds immediately preceding and following a pause event would result from distinct phases of enzyme motion, and these would not a priori be the same. However, velocities entering and leaving a pause are experimentally indistinguishable (our data and those of Adelman et al., 2002).

We propose instead that ubiquitous pausing represents a conformational change in RNAP leading to a short-lived, catalytically deficient state. This rearrangement could correspond to a shift in position relative to the catalytic center of the 3′ end of the RNA, of key residues of the β or β′ subunits, of the catalytic metal ions, of specific bases in the template DNA strand, or some combination of these components. However, the sum of motions associated with the proposed rearrangement must be small and cannot lead to a displacement of the DNA relative to the C terminus of the β′ subunit of RNAP by a distance >1 Å (Figure 6). Evidence exists for a number of mechanical transitions that potentially satisfy these requirements. First, crosslinking studies reveal that when RNAP is initially halted at an arrest site, the RNA 3′ end is displaced by at least 15 Å from the active site (Markovtsov et al., 1996). This "fraying" of the RNA from the DNA template may prevent the correct alignment of incoming NTPs (Artsimovitch and Landick, 2000). Second, different crystal structures show the so-called "bridge helix" of the β subunit in either a straight, or a "kinked," conformation (Gnatt et al., 2001). One of these structures may impede catalysis. Third, the "bottom claw," or clamp, domain is capable of relatively large-scale motions, which may occur during initiation of transcription (Darst et al., 2002). A partial opening of the claw could lead to a misalignment of the reactive groups (Erie, 2002; Landick, 2001).

Relation of Ubiquitous Pauses to the Unactivated Intermediate

The unactivated intermediate has been proposed to explain the kinetics of misincorporation and nucleotide addition (Holmes and Erie, 2003; Erie et al., 1993; Foster et al., 2001) and as a common precursor to different classes of regulatory pause, as well as to termination and arrest (Figure 7) (Artsimovitch and Landick, 2000; Palangat and Landick, 2001; Erie, 2002). An unactivated intermediate may form in response to sequences encountered in the RNA:DNA hybrid and downstream DNA (Landick, 2001). Furthermore, the unactivated intermediate does not appear to be backtracked. One study of the HIV-1 pause using RNAPII concluded that the formation of the unactivated intermediate precedes backtracking (Palangat and Landick, 2001). In addition, the lifetime of the unactivated intermediate was shown to vary with NTP concentration. In another study, the half-life of a hairpin-stabilized pause (*his*) was shown to decrease from 47 s to ~3 s upon addition of antisense oligonucleotides that prevent hairpin formation (Artsimovitch and Landick, 2000). This finding suggests that the short-lived unactivated intermediate (3 s) persists even after disruption of the long-lived pause signal (Figure 7).

It is tempting to speculate that ubiquitous pauses reflect the unactivated intermediate state. Consistent with this, we measured an average rate of entry into

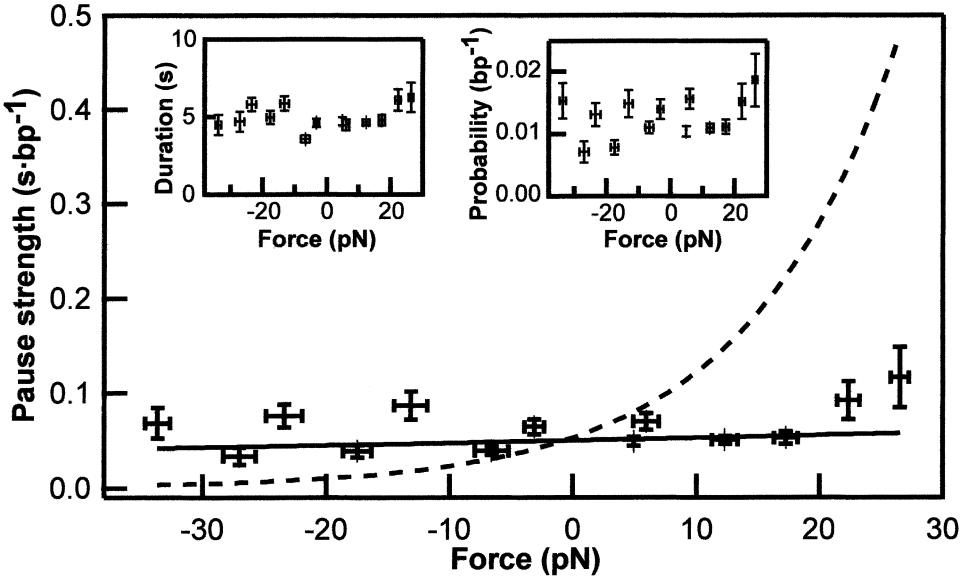

Figure 6. Pause Statistics as a Function of Load

Pause strength, pause duration (left inset), and pause probability (right inset) versus applied load. Values are displayed with errors estimated as (mean ± SE). The dashed curve displays the relationship expected if pausing were caused by a 1 bp backtracking motion ($D = 1$). The solid curve shows the best fit of the data to the single barrier model, yielding $D = 0.06 \pm 0.03$ bp (0.2 Å).

pause (defined as the number of pauses per second of transcription) of ~0.1 s^{-1} (6 min^{-1}), which is comparable with reported rates of entry into the unactivated state at specific sequences (Erie et al., 1993).

The *his* and *ops* pause signals, two mechanistically distinct regulatory pauses that have been studied by biochemical methods (Artsimovitch and Landick, 2000), exhibit pause strengths comparable to the values reported here under similar solution conditions, NTP concentrations, and temperature (data not shown). These findings are consistent with a model in which both regulatory pauses arise from the unactivated intermediate, but at saturating NTP concentrations and in the absence of regulatory factors, only the unactivated intermediate can accumulate.

Proposed Roles of Ubiquitous Pausing In Vivo

If ubiquitous pauses are a manifestation of the unactivated intermediate, they would constitute a useful target for transcriptional regulation (Palangat and Landick, 2001; Erie, 2002). Control of the unactivated intermediate could provide a means to modify simultaneously the response of RNAP to several intrinsic regulatory signals. For example, downregulation of pauses through the binding of accessory proteins, such as the antitermination factor λQ (Santangelo et al., 2003), could increase the rate of elongation and decrease the efficiency of intrinsic terminators and regulatory pauses.

During transcription of mRNA, ubiquitous pauses may serve to couple transcription to translation by limiting the average rate of mRNA synthesis to more closely match that of polypeptide synthesis (~50 bp/s), which is significantly slower than the maximal transcriptional rate (~90 bp/s) obtained during rRNA synthesis (Richardson and Greenblatt, 1996, and references therein). We found support for this conjecture by considering the effect of the measured pause statistics on a pause-free rate of 90 bp/s. Assuming a simple off-pathway model, in which entry into the paused state competes with forward progress, the average velocity in the presence of pauses would drop to around 60 bp/s, with one pause every 400–500 bp. Ubiquitous pausing is therefore a sufficient mechanism to couple transcription and translation. During rRNA synthesis, ribosomal antitermination factors that stabilize the TEC (Richardson and Greenblatt, 1996) may increase the elongation rate by suppressing most transient pausing.

Velocity Heterogeneity

A common feature emerging from many single-molecule studies of RNAP is a broader-than-anticipated distribution in the average velocities of individual molecules. In some instances the average velocity of an individual RNAP molecule has been reported to change significantly during the course of transcription, either at a low substrate concentration (Davenport et al., 2000) or with a mutant polymerase (Adelman et al., 2002). These data suggest that polymerase molecules could switch between different translocation speeds. We did not observe any such velocity state switching in our assays; the rates of advance between pauses were remarkably uniform and were represented by a single peak in the velocity distribution (Figure 1). However, the global distribution of transcription velocities (Figure 2) is too broad to arise merely from the statistical spread expected from a sample of elongation complexes moving at a single average rate (*H*-test; $p < 0.001$). We conclude that individual RNAP molecules, under the conditions of our experiments, must exist in distinct catalytic states that are individually stable or that interconvert only on long timescales (2–18 min).

Similar velocity heterogeneity has been noted in sev-

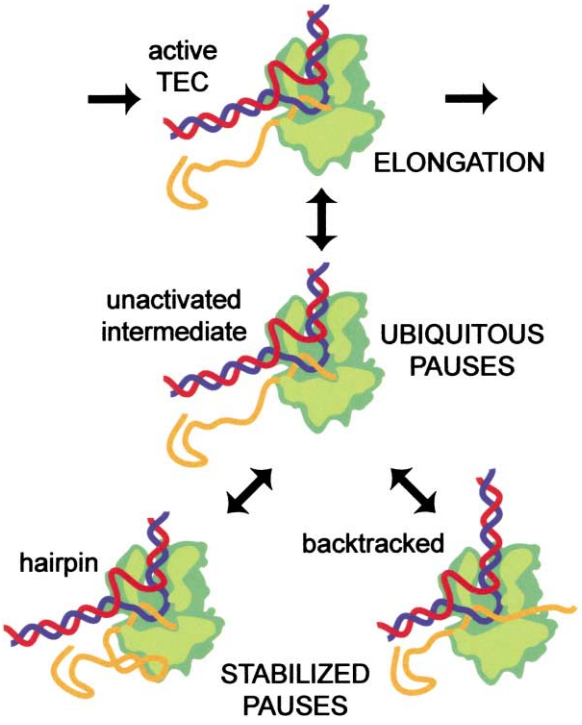

Figure 7. Unified Model for Pausing Based on the Unactivated Intermediate State

RNAP in the elongation phase of transcription (top; horizontal arrows denote the normal elongation pathway) can enter a transient kinetic state known as the "unactivated intermediate" (middle), which undergoes a minor rearrangement of the enzyme active site (note the frayed 3′ end of the nascent RNA). The formation of the unactivated intermediate precedes entry into pause states associated with either RNA hairpin formation or enzyme backtracking (bottom; vertical arrows denote transitions among these states).

eral previous single-molecule studies of transcription (Tolić-Nørrelykke et al., submitted; Yin et al., 1994, 1995; Wang et al., 1998) and has also been inferred from bulk biochemical studies (Erie et al., 1993; Foster et al., 2001; Matsuzaki et al., 1994). However, Adelman et al. (2002) reported a comparatively low heterogeneity in transcription rates of an epitope-tagged polymerase, which they concluded was consistent with a single catalytic state. Unfortunately, because no quantitative measure of the variation in speed between pauses was reported, a direct comparison with our data is not feasible. We further note that the variance in the velocity distribution reported by Adelman et al. (2002) exceeded the value attributable to their measurement error, implying some additional source of variance whose origin remains a matter of speculation.

Several possible explanations could account for the velocity heterogeneity found here. Transcriptional or translational errors during the synthesis of RNAP could lead to the replacement of catalytically relevant residues. Posttranslational modifications could alter the activity of individual polymerase molecules. Alternatively, individual molecules could adopt slightly different conformations, due to misfolding or misassembly of polymerase subunits. Importantly, the heterogeneity in speed cannot be attributed to the application of force because it is seen under a wide variety of loading conditions, including both assisting and hindering forces (our data) and also in experiments on RNAP performed at negligible loads (Tolić-Nørrelykke et al., submitted).

Analogous heterogeneity in enzyme activity has been observed in many of the enzymes studied thus far at the single-molecule level, including lactate dehydrogenase (Xue and Yeung, 1995), the hairpin ribozyme (Zhuang et al., 2000, 2002), and DNA polymerase (Maier et al., 2000; Wuite et al., 2000). Further investigation, both biochemically and at the single-molecule limit, will be required to determine the origin and possible biological relevance of such heterogeneity. However, our central conclusion, that ubiquitous pauses are unaffected by load, holds regardless of the specific source of velocity heterogeneity.

Force-Velocity Relationship

Force-velocity measurements demonstrate that translocation by RNAP can be halted only by large hindering forces ($>$27 pN). Because ubiquitous pauses are not affected by load, we conclude that stalling is a distinct phenomenon mediated by an independent reaction pathway. The $\sim$9 bp (3 nm) distance parameter obtained from the single barrier model (Supplemental Data available on *Cell* website) may correspond to the backtracking of the polymerase along DNA, leading to entry into an inactive state.

An alternative hypothesis is that stalling at high load is caused by a mechanical perturbation of the TEC structure. If the decrease in velocity resulted from a force-induced, partial denaturation of the enzyme, then high hindering or assisting loads would likely exert comparable effects on transcriptional velocity. The asymmetry between assisting and hindering forces observed in the *F-v* relation, however, suggests that stalling results instead from a directionally specific movement of RNAP relative to DNA (Figure 3). While our data are consistent with a model in which this motion corresponds to backtracking, we cannot rule out some other directionally dependent mechanical perturbation of the TEC, such as a large-scale ($>$3 nm) conformational change within the enzyme, or a reconfiguration of the protein-nucleic acid contacts.

Conclusions

Improvements in optical trapping instrumentation and single-molecule transcription experiments permit the acquisition of high-resolution recordings of single RNAP molecules transcribing under both assisting and hindering loads. Transient pauses occur ubiquitously throughout the gene studied (a gene whose sequence was nominally devoid of regulatory pauses), over a wide range of intervals associated with at least two time constants (1.2 s, 6.0 s), plus some long-lived outliers. Ubiquitous pauses do not arise from backtracking motions, as evidenced by their insensitivity to load. We propose instead that pauses may develop from small rearrangements near the enzyme active site, possibly corresponding to the unactivated intermediate state believed to precede long-lived pauses, arrest, and termination.

The presence of an unactivated intermediate in the

pausing pathway leads to several testable predictions. First, because the lifetime of the unactivated intermediate is dependent upon NTP concentration (Palangat and Landick, 2001), the mean lifetimes of ubiquitous pauses should depend upon concentration in a corresponding manner. Second, because the short-lived unactivated state is an intermediate in the pathways to the longer-lived pause states induced by regulatory sequences (Figure 7), the lifetime distribution for such pauses should reflect the rate constants for both transitions. DNA templates with imbedded regulatory sites would offer an opportunity to study both short- and long-lived pause states side by side. Finally, the disruption of a regulatory pause may reveal the presence of the underlying unactivated intermediate. This may be accomplished, for example, by the application of load to the enzyme to prevent rearward motion at a backtracking pause (e.g., *ops*), or the application of load to the nascent RNA to prevent secondary structure formation at a hairpin pause (e.g., *his*).

Experimental Procedures

Avidin-Coated Beads

Avidin-coated 500 nm diameter polystyrene beads (Bangs Laboratories) were prepared as described in Yin et al. (1994), except that beads were purified by repeated pelleting and resuspension rather than gel filtration. Bead concentration was determined by comparing turbidity at 500 nm with a standard curve.

DNA Template

Transcription experiments employed DNA templates generated from plasmid pRL732 by 28 PCR cycles with an XL PCR kit (Applied Biosystems). The plasmid was constructed by inserting the 2659 bp BstXI to BstEII fragment from plasmid pRL574 (Schafer et al., 1991) into the corresponding sites in pRL777. pRL777 was produced by inserting the 300 bp SacI-SpeI fragment of pCL102B (Chan and Landick, 1997) into the SacI-XbaI sites of pRL651 (Yin et al., 1999). The transcription template contains 1098 bp of DNA upstream from the T7 A1 promoter, which is followed by 3792 bp of the *E. coli rpoB* gene in the sense direction. For assisting force experiments, the upstream primer was 5′-digoxigenin-labeled (all primers from Operon), whereas for hindering force experiments the downstream primer was digoxigenin-labeled.

Stalled RNAP Ternary Complexes

Transcription elongation complexes stalled at A29 were prepared and purified as previously described (Yin et al., 1994), except that a biotin-labeled *E. coli* RNAP derivative, modified by adding residues of biotin carboxyl carrier protein to the C terminus of the β′ subunit, was used (Tolić-Nørrelykke et al., submitted). Beads were attached to the biotin label by incubating 3-fold excess avidin-coated beads with the stalled complexes, which ensured that fewer than 5% of the beads had multiple complexes, assuming independent binding.

Optical Trap

The optical trap employed for these experiments has been previously described (Wang et al., 1997). The instrument was enhanced by adding a feedback-equipped, piezoelectric stage (Physik Instrumente), which affords three-dimensional positioning with an accuracy and precision on the order of 1 nm. The stage was also incorporated into a force-feedback loop, which permitted recording motion over many micrometers while maintaining a constant force. The instrument was run by a custom software suite implemented in LabView (National Instruments).

Flow Cell Preparation for Trapping Experiments

Flow cells (~30 μL) were assembled from #1½ glass coverslips (Corning) attached to a microscope slide (Corning) by two parallel strips of double-sticky tape, arranged to form a channel (~0.5 cm wide) running across the narrow axis of the slide. Prior to assembly, coverslips were sonicated in a saturated KOH-ethanol solution, rinsed with deionized water, and dried in an oven. Flow cells were first incubated for 40 min with 20 μg/mL of antidigoxigenin polyclonal antibody (Roche Molecular Biochemicals) dissolved in PBS (Mallinckrodt), then washed with 500 μl transcription buffer (50 mM HEPES or 20 mM Tris, [pH 8.0], 130 mM KCl, 4 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 20 μg/mL heparin). Nonspecific binding of beads was reduced by washing the flow cells with 200 μl of 3 mg/mL bovine serum albumin (Calbiochem) in transcription buffer and incubating for 20 min. The flow cells were then perfused with 200 μl of bead-labeled, stalled transcription complexes (10 pM), and incubated for 15 min, followed by a final wash of 600 μl transcription buffer. Flow cells were kept at 4°C until used. Stalled complexes were restarted by perfusion with transcription buffer supplemented with 1 mM NTPs (Roche Molecular Biochemicals) and an oxygen scavenging system consisting of 50 units/mL glucose oxidase (Calbiochem), 10 units/mL catalase (Calbiochem), and 450 mg/mL glucose (Sigma), to prevent photo damage from the optical trap (Neuman et al., 1999). All experiments were performed in a low-vibration, temperature-regulated clean room at 21.1 ± 0.5°C.

Force Clamp Data Collection

After restarting the stalled ternary RNAP complexes, the bead was captured in the optical trap. The location of the surface of the flow cell was found by raising the coverslip until the trapped bead contacted it and was moved slightly out of the optical trap in the axial direction. As the bead moved through the trap, the intensity of the scattered light displayed a reproducible peak that was a fixed distance from the surface of the coverslip, permitting surface height determination to within 10 nm (data not shown). Once this position was found, the stage was lowered so that the trapping equilibrium position was centered 300 nm above the surface. Vertical motion of the stage was corrected for the mismatch in index of refraction at the interface of the coverslip and sample, which results in a scaling factor of 0.8 between the stage motion and the change in trap height (data not shown). The stage was then moved horizontally back and forth to generate a DNA force-extension (stretching) curve (Wang et al., 1997), which was used to determine the tether attachment point (Wang et al., 1997). During an experimental run, the *x*- and *z*-position signals were low-pass filtered at 1 kHz, digitized at 2 kHz by means of a multifunction DAQ board (National Instruments), and boxcar-averaged over 40 points to generate a 50 Hz signal that was used to control the motion of the stage.

Data Analysis

Motion of the stage was corrected for the elastic compliance of DNA (Wang et al., 1997) and the displacement of the bead from the trap center to recover the contour length of the DNA tether as a function of time. Motion of the RNAP along the template was determined by subtracting the initial tether length from the computed DNA contour length and converting to bp using a conversion factor of 0.338 nm/bp. Precision of the position measurement was limited largely by the Brownian motion of the bead, which is governed by the combined stiffnesses of the trap and DNA tether. Uncertainty in position varied from 10 bp at 5 pN to 4 bp at forces >35 pN. Absolute accuracy of the measurements was limited by stage drift. The average drift rate of 0.6 bp/s was low compared with the average transcription rate of ~10 bp/s. Over the duration of a lengthy run, however, the accumulated error could be as much as 200 bp. There was also an absolute position uncertainty associated with the initial centering of the tether and the dispersion in bead size (~10%), which we estimated as 75 bp. The time-dependent position of the RNAP molecule along the template was smoothed with a 2nd order Savitzky-Golay filter (Press et al., 1992) with a time constant of 2.5 s and differentiated to generate the instantaneous velocity. The velocity distribution is broader than the noise distribution due to the stochastic properties of the motion (Schnitzer and Block, 1995). Pause detection was largely independent of the exact choice of threshold velocity; using a threshold value of one or two standard deviations from the pause or velocity peak, instead of half the run velocity, changed pause statistics only slightly and did not affect the overall conclusions. The population distribution of transcription

velocities was compared to the properties of individual records using the parameter-free Kruskal-Wallis, or *H*-test (Sokal and Rohlf, 1969). Data reduction and analysis were performed with custom software programmed in Igor Pro (Wavemetrics).

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